# ON THE HARDENING OF THE CHORION OF THE FISH EGG AFTER FERTILIZATION. III. THE MECHANISM OF CHORION HARDENING IN ORYZIAS LATIPES

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Shortly after fertilization or parthenogenetic activation, soft chorions of fishes are transformed into rigid structures mechanically non-elastic, and chemically resistant. This transformation has attracted much attention particularly in recent years (Nakano, 1956; T. S. Yamamoto, 1957; Ohtsuka, 1957; Zotin, 1958; Rothschild, 1958), and some interesting facts on substances participating in the phenomenon have come to our knowledge. However, the mechanism by which fish chorions are hardened is still a matter of speculation. In an earlier paper of this series (Ohtsuka, 1957), the possibility was suggested that in *Oryzias* egg the process underlying chorion hardening involves an oxidation process. This hypothesis seems to be provided further support in the present paper in which there are presented data on the effects of various chemical agents on the chorion hardening.

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## Material and Methods

The ripe unfertilized eggs of the fresh-water fish, *Oryzias latipes,* were used as material. They were taken from matured females and kept in isotonic Ringer's solution. The overripe eggs of which chorions had slightly separated from the egg surface were discarded. The Ringer's solution used here had the following constitution: *M*/7.5 NaCl 100 parts + *M*/7.5 KCl 2.0 parts + *M*/11 $CaCl_2$ 2.1 parts whose pH was adjusted to 7.3 by adding $NaHCO_3$ (T. Yamamoto, 1944). Sperm suspension was also prepared in Ringer's solution.

Treatments with chemical agents were carried out before or after fertilization at room temperature (22–27.5° C.). In the pretreatment, after some lengths of exposure, aliquots of the eggs were put back into Ringer's solution and then inseminated. Besides, in a few experiments insemination was done in the presence of reagents. The effect was examined with regard to chorion hardening. This is possible to judge indirectly from the degree of chorion elevation; it is generally accepted that the chorion is low when hardening is produced by the reagent whereas it elevates very highly under inhibitory condition. On this account the volume of elevated chorions was calculated 50 minutes after fertilization, according to T. Yamamoto's formula (1940). But the above criterion was found to have its

exceptions as described in the text. Therefore, in all the cases, chorions were torn with glass needles for the determination of their stiffness after volume measurement.

## Results

### *I. Oxidizing agents*

The possibility that oxidation takes part in chorion hardening makes it of interest to test whether hardening is caused by means of oxidizing agents. Experiments were conducted on the action of potassium ferricyanide, sodium tetrathionate, iodine, hydrogen peroxide, sodium periodate, chromic acid and potassium dichromate. All the agents were dissolved in Ringer's solution except for periodate. This was made up in Ca-free Ringer's solution to avoid precipitation by calcium.

When unfertilized eggs were subjected to these solutions (pH 7.3) for periods of 15 to 30 minutes, chorion hardening took place in the agents other than chromic acid. Such eggs with hard chorions exhibited no visible cortical changes during each treatment. Here it should be added that some of the eggs were partheno-

Table I

*Effect of the presence of oxidizing agents on the chorion changes of cadmium-pretreated eggs at fertilization. Temp. 22.7° C.*

| Agents | Volume of chorion (cu. mm.) | Chorion hardening |
|---|---|---|
| No treatment | $1.44 \pm 0.018$ | — |
| Potassium ferricyanide ($2 \times 10^{-3}$ *M*) | $0.99 \pm 0.015$ | + |
| Sodium tetrathionate ($5 \times 10^{-3}$ *M*) | $0.99 \pm 0.020$ | + |
| Iodine in potassium iodide ($2.5 \times 10^{-4}$ *M*) | $0.98 \pm 0.017$ | + |
| Hydrogen peroxide (1.5%) | $1.02 \pm 0.014$ | + |
| Sodium periodate ($4 \times 10^{-4}$ *M*) | $0.98 \pm 0.012$ | + |
| Chromic acid ($1 \times 10^{-2}$ *M*) | $1.40 \pm 0.016$ | ± |
| Potassium dichromate ($2 \times 10^{-4}$ *M*) | $0.97 \pm 0.011$ | + |

genetically activated in both hydrogen peroxide and tetrathionate. In a chromic acid solution the chorion remained unchanged even after more prolonged exposure. It was found, however, that if the solution is acidic, chromic acid, though less effective, is capable of inducing hardening; at pH 6.6 it required a lapse of 60 minutes or more until the chorion became hardened. Then, Ringer's solution of chromic acid with this pH value was employed in the following experiments, but did not give any appreciable effect because of its feeble hardening ability. Treatments with oxidizing agents were carried out soon after fertilization. It was found that except in the case with chromic acid, all the other agents bring about the lowering of chorion elevation as a result of the acceleration of the hardening which follows fertilization.

Table I shows a typical result of experiments in which eggs immersed in a cadmium chloride-Ringer's solution ($2.5 \times 10^{-3}$ *M*) for 10 minutes were transferred to the solutions of oxidizing agents immediately following insemination in Ringer's solution. Cadmium treatment is known to abolish chorion hardening (Ohtsuka, 1957). It will be seen, however, that in these eggs except the chromic acid-treated ones, the chorions elevated were lower so that there developed a narrow perivitelline space.

It is of special interest that chromic acid is less effective than any of the other agents in inducing hardening. The discussion on this point will be presented later.

*II. Reducing agents*

Experiments were performed using the following reducing agents to determine whether their action on chorion hardening would be reverse to that of oxidizing agents: sodium sulfide, potassium cyanide, sodium thiosulfate, sodium sulfate, sodium thioglycolate, ammonium sulfate and potassium ferrocyanide. These agents were dissolved in appropriately diluted Ringer's solution so as to become osmotically equivalent to *M*/7.5 NaCl, and prepared in concentrations to contain the effective amount of calcium, since the deficiency of the latter blocks hardening of the chorion (Ohtsuka, 1957). The pH of all the solutions was made to 7.3 with HCl or NaOH.

Freshly inseminated eggs were put into each isotonic solution of the reducing agents. Here in both solutions of sulfide and cyanide the chorions were incapable of hardening, but such an effect of the remaining agents was observed only in a

TABLE II

*Effect of the presence of reducing agents on the chorion changes at fertilization. Temp. 24.1° C.*

| Agents | Pretreatment (min.) | Volume of chorion (cu. mm.) | Chorion hardening |
|---|---|---|---|
| No treatment | — | 1.05 ± 0.014 | + |
| Sodium sulfide ($2 \times 10^{-2}$ *M*) | — | 1.07 ± 0.017 | − |
| Potassium cyanide ($3.3 \times 10^{-2}$ *M*) | — | 1.07 ± 0.012 | − |
| Sodium thiosulfate ($6.6 \times 10^{-2}$ *M*) | 5 | 1.06 ± 0.023 | − |
| Sodium sulfate ($6.6 \times 10^{-2}$ *M*) | 5 | 1.07 ± 0.018 | − |
| Sodium thioglycolate ($9 \times 10^{-2}$ *M*) | 30 | 1.06 ± 0.015 | − |
| Ammonium sulfate ($6.6 \times 10^{-2}$ *M*) | 5 | 1.06 ± 0.020 | − |
| Potassium ferrocyanide ($4 \times 10^{-2}$ *M*) | 10 | 1.06 ± 0.013 | − |

few cases (*e.g.*, in 2 of 9 experiments with thiosulfate). If, however, fertilization was done in the presence of these agents with which the eggs had previously been treated for some periods (5–30 minutes), hardening always failed to occur. Reducing agents were found to be effective in high concentrations. One of the representative results is tabulated in Table II, where it can be seen that under the influence of reducing agents the chorions did not markedly elevate in spite of the inhibition of their hardening. And they were resistant to osmotic pressure of the perivitelline fluid. The factor inhibiting further elevation of the soft chorion in these circumstances is not known. On the other hand, it was impossible to prevent chorion hardening when eggs were inseminated in Ringer's solution after any length of pretreatments. Furthermore, the chorions once hardened could not be converted into a soft condition by treatments with reducing agents.

*III. Sulfhydryl compounds*

It has not yet been established on the nature of the chorion substrate undergoing hardening in fish eggs. The author (1957) was interested in SH groups,

TABLE III

*Effect of 5 minutes' pretreatments with mercaptide-forming agents on the chorion changes at fertilization. Temp. 25.4° C.*

| Agents | Volume of chorion (cu. mm.) | Chorion hardening |
|---|---|---|
| No pretreatment | 1.05 ± 0.014 | + |
| Mercuric chloride ($2.5 \times 10^{-5}$ *M*) | 1.47 ± 0.022 | − |
| p-Chloromercuribenzoate* | 1.48 ± 0.016 | − |

* Saturated in Ringer's solution.

anticipating that their reactivity might contribute to the chorion hardening. To elucidate this point, two series of experiments were undertaken, one with a mercaptide-forming agent and the other an alkylating agent. Preliminary study by a staining method on the composition of soft chorion seems to demonstrate the presence of SH groups (presumably protein bound-SH groups); no nitroprusside reaction was given, while it was stained with coloured SH reagent (method of Mescon and Flesch), even when this staining was applied after fixing the chorion with 2 per cent trichloroacetic acid.

In the first experiments, unfertilized eggs were placed for 5 minutes in two mercaptide-forming agents in Ringer's solution, mercuric chloride and p-chloro-

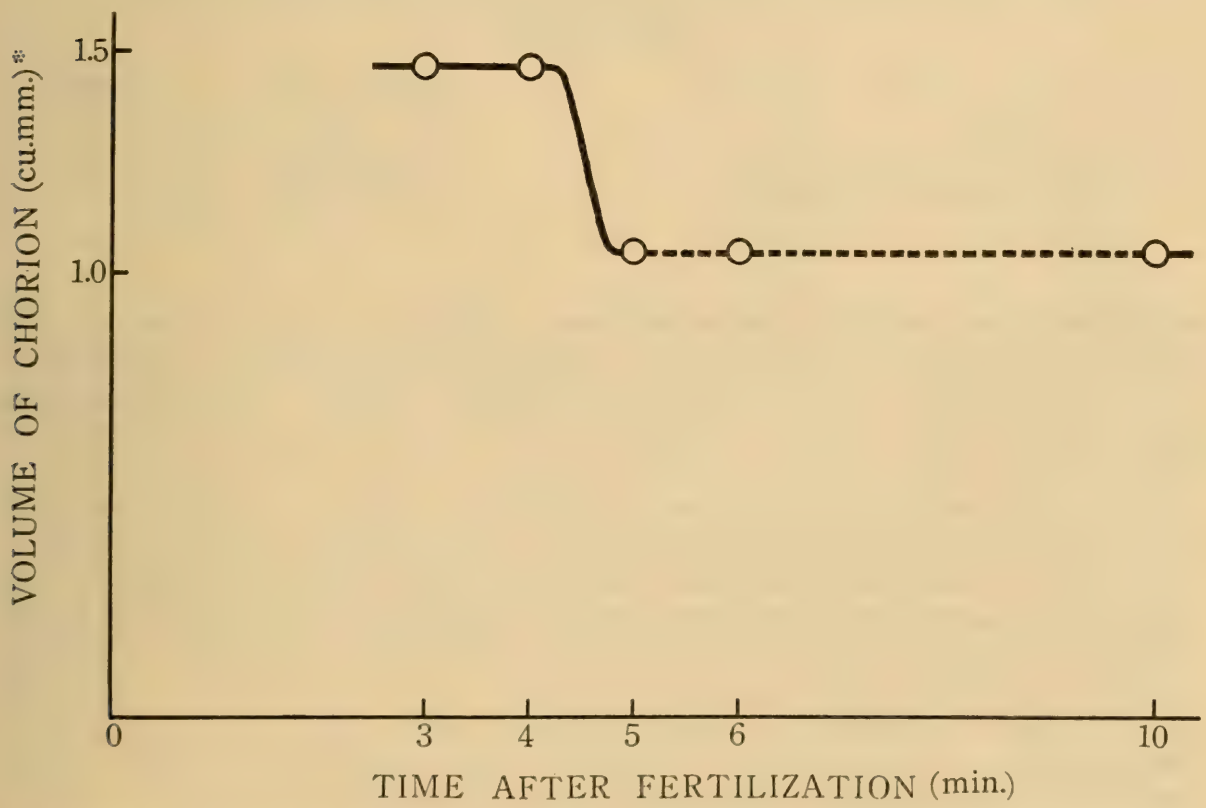


FIGURE 1. Changes in chorion volume in PCMB-Ringer's solution at different times after fertilization. Dotted line shows a period during which the chorion was burst by osmotic pressure of perivitelline fluid.

* Points (5, 10 and 15) of chorion volume in Figure 2 of the previous paper (Ohtsuka, 1957) should be corrected to 0.5, 1.0 and 1.5, respectively.

mercuribenzoate (PCMB). (Lapse of more than 5 minutes in these solutions caused the migration of cortical alveoli towards the animal pole without activation of the egg.) Insemination was carried out in Ringer's solution immediately after the pretreatment. In both cases, chorions were deprived of hardening and there occurred a marked elevation followed by bursting (Table III). This inhibition is due to the blocking of SH groups as shown in the following experiment. According to Anson (1945), the combination of mercury with SH groups can be removed by cyanide. Then, eggs which had been treated with mercaptide-forming agents for 5 minutes as above were consecutively transferred to potassium cyanide-Ringer's solution ($3.3 \times 10^{-2}$ *M*, pH 7.3) prior to insemination. When these eggs were fertilized in Ringer's solution after 5 minutes, reversal of the inhibition was observed. The chorion showed a normal degree of elevation, owing apparently to the occurrence of its hardening.

The failure to harden was also produced by exposing eggs to each mercaptide-forming agent after insemination, and their inhibitory effect could similarly be cancelled upon subsequent treatment with potassium cyanide. Figure 1 represents the results of a typical experiment with PCMB at 25.8° C. If PCMB was applied within 10 minutes after fertilization, further hardening did not proceed

TABLE IV

*Effect of the presence of alkylating agents on the chorion changes at fertilization. Temp. 26.2° C.*

| Agents | Volume of chorion (cu. mm.) | Chorion hardening |
|---|---|---|
| No treatment | $1.05 \pm 0.012$ | + |
| Iodoacetic acid ($3.3 \times 10^{-2}$ *M*) | $1.48 \pm 0.017$ | − |
| Chloroacetophenone* (0.05%) | $1.47 \pm 0.024$ | − |

* Saturated in Ringer's solution.

and the elevated chorions retained softness. It should be noted, however, that when this treatment was made about 5 minutes after fertilization or later, such soft chorions became incapable of elevating highly. Unlike the cases of reducing agents described above, they were burst by an osmotic pressure of the perivitelline fluid.

In the next experiments, eggs were subjected to treatment with iodoacetic acid or chloroacetophenone. These alkylating agents were made up in Ringer's solution (pH 7.3). It was found that either short treatment of unfertilized eggs is not inhibitory to chorion hardening when they are subsequently inseminated in Ringer's solution. If the duration of these exposures was long, in the case of chloroacetophenone fertilized eggs underwent cytolysis and the iodoacetic acid-treated eggs lost their fertilizability. When, however, both agents were applied after insemination, the chorions failed to harden. Eggs exposed to alkylating agents soon after fertilization formed considerably high chorions (Table IV).

### *IV. Urea and lithium bromide*

The above experiments clearly indicate that SH groups primarily participate in hardening of the chorion. They might be expected to be available as a possible

donor of hydrogen bonds. If this is really the case, the latter thus given would be responsible for hardening. According to Nakano (1956), the hydrogen bonds are concerned in the mechanical properties of the chorion of *Oryzias*. Attempts were thus made with urea and lithium bromide, which are known to cleave hydrogen bonds. The maximum concentrations at which they were tested were prepared in $3.3 \times 10^{-2}$ *M* both in Ringer's solution. Eggs were treated before or after fertilization. But neither reagent prevented chorion hardening, even in those cases where unfertilized eggs were exposed for 60 minutes and then inseminated therein. Thus it seems that hydrogen bonds play no role in giving rise to a chorion hardening.

### *V. Aldehydes*

In a staining study, the soft chorion was further found to be periodate-Schiff-positive (Lillie's method), which was blocked by the acetylation technique of McManus. From this result it is evident that a polysaccharide having $\alpha$-glycol groups impregnates the chorion and produces aldehydes on oxidation (*cf.* Lison, 1953). The question arises whether such aldehydes take part in hardening of

TABLE V

*Effect of the presence of aldehydes on the chorion changes of cadmium-pretreated eggs at fertilization. Temp. 27.3° C.*

| Agents | Volume of chorion (cu. mm.) | Chorion hardening |
|---|---|---|
| No treatment | 1.48 ± 0.017 | − |
| Formaldehyde (0.03%) | 0.97 ± 0.014 | + |
| Acetaldehyde (0.5%) | 0.98 ± 0.018 | + |
| Acrolein (0.05%) | 1.01 ± 0.012 | + |
| Benzaldehyde (0.2%) | 0.99 ± 0.015 | + |

the chorion. To clarify this point, the effect of aldehydes, such as formaldehyde, acetaldehyde, acrolein and benzaldehyde, was investigated. Ringer's solutions of these agents were adjusted to pH 7.3 by adding NaOH.

The results obtained were essentially the same as those with oxidizing agents noted already. The chorions became hardened without egg activation in the presence of the agents other than acrolein. In the latter solution, almost all eggs were parthenogenetically activated 2–3 minutes after their immersion and there took place faster chorion hardening than in the control. Table V illustrates that aldehydes bring about the formation of low chorion when cadmium-pretreated eggs are treated after fertilization in the same way as in the cases of oxidizing agents.

## DISCUSSION

As shown in the foregoing pages, the chorion fails to harden under the influence of certain reducing agents. Very high concentrations of the agents are characteristic of this inhibition, suggesting the existence of an antagonistic force responsible for hardening. It has been reported in *Oryzias* egg that a phospholipid in the perivitelline space, which is originated from the cortical layer itself following fertilization or artificial activation, is of prime importance in giving rise to the

chorion hardening, the action of which is regarded as oxidative in nature (Ohtsuka, 1957). Therefore it is most probable that reducing agents inhibit hardening by interfering with this oxidative effect. On the other hand, the oxidizing agents, potassium ferricyanide, sodium tetrathionate, iodine, hydrogen peroxide, sodium periodate, chromic acid and potassium dichromate, were found to have the hardening capacity. The effectiveness of these agents in hardening the chorion is probably due to oxidation. We shall return to this aspect later.

Similar results have been obtained with sea urchin eggs by Motomura and Hiwatashi (1954), who tested the effects of various chemicals on the hardening of the fertilization membrane, and found that the action of reducing agents is opposite to that of oxidizing agents; the former inhibits membrane hardening whereas the latter accelerates it. They offered an explanation that hardening is concerned with polymerization to which both agents are sensitive in a reverse manner. But, as just discussed, the present results can be reasonably interpreted from an oxidative point of view. Still this does not preclude the possibility of polymerization in the hardening of the chorion.

In view of these considerations, it may be allowable to conclude that the oxidation process is involved in the process whereby *Oryzias* chorion is converted into rigid structure. And it appears that it takes part in the initial stage of the hardening process, as is indicated by the fact, for example, that the chorion became hardened even when treatment with sodium periodate was carried out for 10 minutes, during which no changes were visible, and then kept in Ringer's solution. This conclusion is, however, contradicted by Nakano's observation (1956) that colloidal substances, such as gum arabic, gum tragacanth, albumin and gelatin, are capable of hardening *Oryzias* chorion. These substances are unfortunately non-oxidative in chemical nature, their mechanisms remaining obscure.

In a more recent study, Zotin (1958) has found a substance indispensable for the chorion hardening in the perivitelline space of some salmonid eggs. According to him, this substance, which was termed the hardening enzyme, is secreted from the cortical layer, but not from the alveoli present there, upon fertilization or activation, and plays an active role in hardening the chorion. Thus there is a considerable resemblance between phospholipid in *Oryzias* egg and the hardening enzyme of salmonid eggs, particularly with respect to their source and behaviour. Furthermore, Zotin described that hardening of the chorion in salmonids, which is due to polymerization of some substances, is blocked by oxygen deficiency.

It has been shown in the present paper that the SH groups (presumably protein bound-SH groups) of the chorion are essentially necessary for hardening, while their role bears no relation to their possible capacity to give hydrogen bonds. In this connection it should be emphasized that the first three of the effective oxidants noted above, potassium ferricyanide, sodium tetrathionate, and iodine, all oxidize SH groups (Anson, 1945). A similar action, though less reliable, is known about hydrogen peroxide (see Hirai, 1957) capable of hardening as well. Hence these strongly suggest an oxidative conversion of SH into S-S; the latter thus formed may be responsible for chorion hardening. This change must proceed beyond the reversible stage, because a hard chorion is not returned to the soft condition by reducing agents so far as the present study is concerned. Additionally, there is evidence that a chorion polysaccharide having $\alpha$-glycol groups participates

in hardening. Sodium periodate, another effective oxidant, does change all the available α-glycol groups to aldehydes (*cf.* Lison, 1953). Then, if such a type of oxidation is involved in the chorion hardening, it is worth pointing out that chromic acid likewise produces aldehydes in oxidizing the polysaccharide but it seems without action on α-glycol groups (*cf.* Lison, 1953); this may be the reason why chromic acid is less effective in inducing hardening in spite of its strong oxidative capacity. It is therefore likely that in hardening α-glycol groups are subject to oxidation, resulting in the formation of aldehyde groups. Favoring this idea is the fact that the chorions harden by treatments with aldehyde, such as formaldehyde, acetaldehyde, acrolein and benzaldehyde. Thus it may be said that two substances of the chorion, a protein containing SH groups and a polysaccharide which has α-glycol groups, form a complex undergoing hardening.

From the above account it follows that the reactivities of both sulfhydryl and α-glycol groups contribute to the hardening of the chorion. There must occur some profound changes in these two groups, especially with regard to their, and perhaps reciprocal, oxidative reactions. However, an alternative possibility remains, based on the following facts: According to the present data, SH groups become responsible for chorion hardening about 10 minutes after fertilization. It was further shown that when PCMB, which attacks SH groups, is applied about 5 minutes after fertilization, the chorion retaining softness is incapable of elevating very highly, being burst by osmotic pressure of the perivitelline fluid. These observations reveal some preceding change, probably of polysaccharide, and favor the assumption that oxidation concerns first the α-glycol groups only and then it is followed by that of SH. Significant in this respect is a hardening effect of certain aldehydes mentioned already, and moreover evidence is available that aldehydes are able to combine with SH groups (Schubert, 1936), which latter still remain capable of being oxidized (Anson, 1945).

Thus it may be suggested that hardening is due to the combination of at least two factors in the chorion, an SH group, and an aldehyde produced by oxidation of α-glycol group. And the former would undergo oxidation to S-S. There might be an oxidation polymerization through these disulfide bridges. Very interesting is that the reaction between SH groups and aldehydes is related to a skin tanning (Hirai, 1957). Furthermore, it should be mentioned that potassium dichromate capable of hardening is an excellent tanning agent. Considering these, a tanning reaction appears to take place in the chorion hardening. Rothschild (1958) proposed that fish chorions are hardened by the tanning effect of substance which is discharged from the cortical alveoli at fertilization or parthenogenetic activation. The positive effect of the alveolar substance on chorion hardening has been suggested by Nakano (1956) in *Oryzias* and by T. S. Yamamoto (1957) in *Oncorhynchus*. But the unpublished data on *Oryzias* egg show that hardening is independent of the alveolar substance.

## Summary

1. Oxidizing agents, such as potassium ferricyanide, sodium tetrathionate, iodine, hydrogen peroxide, sodium periodate, chromic acid and potassium dichromate, cause hardening of the chorion. Chromic acid is less effective than any of the other agents.

2. In the presence of reducing agents, such as sodium sulfide, potassium cyanide, sodium thiosulfate, sodium sulfate, sodium thioglycolate, ammonium sulfate, and potassium ferrocyanide, the chorions fail to harden.

3. Treatment of unfertilized eggs with mercuric chloride or p-chloromercuribenzoate induces the inhibition of chorion hardening when they are subsequently inseminated in Ringer's solution. The same result is also obtained in the cases where both agents are applied after fertilization. Either inhibitory effect is removed if the treatment is followed by that with potassium cyanide.

4. Iodoacetic acid and chloroacetophenone likewise inhibit hardening when eggs are treated after insemination.

5. Neither urea nor lithium bromide has any effect on the chorion hardening.

6. Aldehydes, such as formaldehyde, acetaldehyde, acrolein and benzaldehyde, are capable of hardening the chorion.

7. Soft chorion is impregnated with a protein containing SH groups, together with polysaccharide which has $\alpha$-glycol groups.

8. It is concluded that the oxidation process is involved in the mechanism of chorion hardening. It is suggested that hardening is due to combination of SH groups with the aldehydes produced by oxidation of $\alpha$-glycol groups.

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